The occurrence of Pierce's disease of grapevines and its control strategies in Taiwan

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ABSTRACT

PD of grapes caused by *Xylella fastidiosa,* a xylem-limited bacterium, has been listed as one of the international quarantine plant diseases. From 2002, the Bureau of Animal and Plant Health Inspection and Quarantine launched a survey project to detect PD in Taiwan. So far, a total of 13,666 grapevines were confirmed to be infected by *X. fastidiosa* via direct isolations and PCR protocols. Other than grapes, the following four plants were confirmed to be the alternative hosts of PD in Taiwan: *Diplocyclos palmatus* (L.) C. Jeffrey, *Ampelopsis brevipedunculata* (Maxim.) Trautv var. *hancei* (Planch.) Rehder., *Humulus scandens* (Lour.) Merr., and *Mallotus paniculatus* (Lam.) Muell.-Arg. The 16S rRNA sequences of PD strains isolated from tissues of grapevines exhibiting PD symptoms collected from 5 counties and 4 alternative hosts were compared with *X. fastidiosa* from other hosts. The phylogenetic trees constructed with neighbour-joining method revealed that *X. fastidiosa* strains from different hosts could be divided into five subgroups and the PD strains from Taiwan were grouped with grapevine and mulberry strains from the Americas. The control strategies recommended for limiting the spread of PD are as follows: (1) to plant the healthy seedlings and eradicate the PD-like grapevines immediately (2) to eradicate the alternative hosts and the weeds that insect vectors favor (3) to control the population of indigenous insect vectors and avoid the invasion of foreign insect vectors.

Keywords: *Xylella fastidiosa,* Pierce's disease of grape, alternative hosts, phylogenetic analysis

INTRODUCTION

Pierce's disease (PD) of grapes, caused by *Xylella fastidiosa*, a xylem-limited bacterium, was described in California in 1892 [14] as Anaheim disease of gapes. The bacterium inhabits only in xylem tissues and requires specific and enriched media for *in vitro* growth [40]. All strains of *X. fastidiosa* are classified as one species but infect different host range. Besides PD, *X. fastidiosa* also causing diseases on citrus [3, 16], pear [19], elm [11], almond [22], oak [4], plum [27], peach [39] and mulberry [18]. Ever since Wells et al.[40] named then xylem-limited bacterium as *X. fastidiosa* in 1987, *X. fastidiosa* has been reclassified into five subspecies according to their differences in genetic makeup, host range, physiology, and biochemistry. They are *X. fastidiosa* subsp. *fastidiosa* for strains of grape, almond, alfalfa, and maple, *X. fastidiosa* subsp. *multiplex* for strains of peach, plum, almond, elm, sycamore, and pigeon grape, *X. fastidiosa* subsp. *pauca* for strains of citrus [31], *X. fastidiosa* subsp. *sandyi* for strains of oleander, daylily, jacaranda, and magnolia [32], and *X. fastidiosa* subsp. *tashke* for strains of *Chitalpa tashkentensis*, a common ornamental landscape plant [26]. However, the last two subspecies have not been officially recognized by the researchers in the community of systematic bacteriology.

Distribution of PD

PD was reported from countries in the Americas including Mexico and the United States in North America, Costa Rica [1] and Venezuela [15] in Central America and Caribbean, and Argentina and Peru [24] in South America. In 1998, Kosovo, former Yugoslavia [2], was the only country that reported a PD incidence in European Continent. In Asian Continent, description of PD-like disorder from China was reported in 2001[7], and PD incidence was confirmed in Taiwan in 2002 [36]. In the southeastern United States, PD is endemic and plays an important role in limiting the development of winery industry. During the period from1994 to 2000, PD destroyed more than 1000 acres of vineyards and caused losses of $30 million in northern California.

The pathogen of PD is *X. fastidiosa*, a gram-negative, non-flagellate, nutritionally fastidious, rod-shaped with rippled cell walls. The cell size was measured 0.2–0.4 μm

in width and 1–3 μm in length (Fig. 1). *X. fastidiosa* only resided in the xylem tissue and the optimal temperature for growth was 26-28℃ and the optimal pH was 6.5-6.9. *X. fastidiosa* grows slowly even in specific and enriched media. At 12-day post-subculture, the opalescent circular colonies reached 1 mm in diameter and were convex with smooth margins in morphology.

Diseased grapevines usually begin with scorch symptoms, necrotic tissue with yellow margins, and followed by the systemic development of leaf scorch symptoms in upper and lower leaves (Fig. 2) [12]. Severely affected grapevines drop leaves early, leaving petioles remain attached to the canes, decline in vigor, followed by stunting, dieback and eventual death in 2-4 years after initial infection [34, 38].

The primary goal of this report was to describe the occurrence of PD and the potential alternative hosts in Taiwan. Moreover, the epidemiological information of PD would be drawn to develop control strategies to reduce the risk of PD in Taiwan.

The occurrence of PD in Taiwan

Table grapes and winemaking grapes were both cultivated in central Taiwan, including Miaoli County, Changhua County, Nantou County, Taichung City. Table grapes including Kyoho, Italia, Honey red and Himrod seedless are competitive to other imported fruits, and gradually become one of the high economic valued fruits in Taiwan; Golden Muscat, Black queen and Muscat Bailey A are for winemaking. In 2002, the Bureau of Animal and Plant Health Inspection and Quarantine launched a survey project to investigate the occurrence of PD in central Taiwan. From 2003 to 2012, a total of 399 vineyards and 13,666 grapevines, including table grapes and winemaking grapes, were confirmed to be infected by *X. fastidiosa* by performing direct isolation and cultivation of the bacterium and PCR [21, 30]; and hence eradicated except those in Changhua County (Table 1).

The survey result showed the morbidity of vineyards conducted from 2002 to 2012 is 19% in Caoton Township, 57% in Zhushan Township, 69% in Waipu District, 93% in Houli District, and 87% in Tonxiao Township (Fig. 3). The winemaking grapes mainly cultivated in Waipu District, Houli District, and Tonxiao Township showed higher morbidity than the table grapes cultivated in areas where they were managed accurately and effectively. In the case of the vineyards in Zhushan, the abandoned vineyards might conserve the pathogen of PD in the diseased plant and the complex vegetation. Once, the alternative hosts were eradicated around the vineyard the insect vectors were forced

to migrate to the adjacent vineyards and spread PD.

The geographic distribution of diseased vineyards in Taiwan.

According to the survey results obtained from central Taiwan, the geographic distribution of diseased vineyards can be summarized as two characteristics: (1) The vineyards in Tonxiao Township, Waipu District and Houli District were typically located in hilly terrain; (2) The vineyards adjacent to the river or valley were found in Zhusan Township, Caoton Township, Zhuolan Township and Xinshe District. These mentioned counties were considered as high risk areas for PD, and the diseased vineyards were almost found in the margin of these counties where they were surrounded by undeveloped mixed forest which favors the survival of the pathogenic bacteria and the insect vectors.

The vineyards in Xinyi Township, Shuili Township and Shigang District have been free of PD incidence. However, the geographical characteristics of vineyards in these counties were similar to those mentioned above; hence they were classified as moderate risk area of PD that need constant survey of PD incidence.

On the other hand, Changhua County, where about 1800 hectares of table grapes were cultivated, has been free of PD according to the detection survey and report of Taichung District Agricultural Research and Extension Station, were classified as low risk area. Based on the following assumptions, we think those in Changhua County might avoid PD explosion: (1) the cultivating area were located in plains, and dense planting cultivation were performed; (2) grapevines are fully replaced approximately every seven years; and (3) immediate eradication of PD-like plants to keep the vegetation clean and simple.

Alternative hosts of PD in Taiwan

X. fastidiosa has a broad host range of native plant species, and many of them appear to be symptomless. In California more than 94 species host plants in 28 families are identified as alternative hosts, including *Acacia longifolia*, *Artemesia vulgaris*, *Avena fatua*, *Chenopodium ambrodioides*, *Fuchsia magellanica*, *Hydrangea paniculota*, *Lolium multiflorum, Marjorana hortensis*, *Poa annua*, *Rosa California*, *Rosemary officinalis*, *Rubus vitifolius*, *Salix* spp., *Veronica* spp. and *Vitis califonica* [9,28].

From 2003 to 2012, about 5,404 plant samples (Table 2) were collected from the proximity of PD-confirmed vineyards in central Taiwan and they were classified as 251 species in 72 families. So far, the result showed 5 *Diplocyclos palmatus* (L.) C. Jeffrey samples in Taichung City and Miaoli county, 8 *Ampelopsis brevipedunculata* (Maxim.) Trautv var. *hancei* (Planch.) Rehder. in Taichung City, Nantou county and Miaoli county, 10 *Humulus scandens* (Lour.) Merr. in Taichung City and Miaoli county, and just 1 *Mallotus paniculatus* (Lam.) Muell.-Arg in Taichung City were confirmed to be the alternative hosts of PD strains based on the direct isolation and PCR detection (Fig. 4). The pathogenic bacteria isolated from four alternative hosts were used for artificial inoculation to healthy grapevines by xylem-infiltration method [8, 13]. The symptomatic leaves appeared from the base near the inoculation sites and moved upwards 1 month post-inoculation.

Upon further analysis, it was revealed that there was a geographic correlation between the distribution of alternative hosts and the diseased grapevines in the vineyard which were indirectly confirmed that the indigenous insects were the vectors in Taiwan. In previous reports, 39 species of Cicadellinae and 5 species of Cercopoidea were confirmed as the vectors of different strains of *X. fastidiosa* in the United States and Brazil [29]. Results of extensive surveys revealed that one *Kolla paulula* (Walker), a xylem-feeding leafhopper, was identified as a candidate insect that may transmit *X. fastidiosa* in central Taiwan [33]. Other than *Kolla paulula* (Walker), we have got positive PCR detection from *Bothrogonia ferruginea* (Fabricius) and *Anatkina horishana* (Matsumura). Nevertheless, the transmission protocol by *B. ferruginea* (Fabricius) and *A. horishana* (Matsunura) has not been tested, and it awaits further investigations for the fulfillment of the Koch's postulates.

Phylogenetic analysis of the PD strains from Taiwan and from the Americas and *X. fastidiosa* from other hosts

The 16S rRNA gene [6,16] sequences of PD strains from Taiwan have been deposited in GenBank under the accession numbers indicated in Table 3 and were used as queries for the similarity search. Twenty sequences of Pierce's disease strains (Table 4) from grapevines and other alternative hosts found in Taiwan were compared with strains from other host plants via multiple sequences alignment by Clustal X program

[17] using *Xanthomonas axonopodis* pv. *citri* strain XCW as an outgroup [5, 10, 36].

The phylogenetic tree was constructed with neighbour-joining method and evaluated by bootstrap analysis for 1000 replicates using MEGA 4 program [37] with the orthologous sequences of XCW as outgroups for phylogenetic analyses [20]. The neighbour-joining trees showed two distinct monophyletic groups of the 31 strains: Group 1 contained 2 PLS strains [35] and group 2 contained the other 29 strains. The strains in group 1 and group 2 were closely related to each other with bootstrap probabilities of 88% and 99% for the 16S rRNA phylogenetic tree. Group 2 can be subdivided into 4 subgroups: GM (grape and mulberry), C (coffee and citrus), PS (peach, pecan, plum and sycamore) and O (oleander) (Fig. 5). The PD strains from grapevines and confirmed alternative hosts in Taiwan were grouped with American PD and mulberry strains that belonged to the subspecies *fastidiosa* [31] which demonstrated that the pathogens might exist in indigenous alternative hosts and from which the indigenous insect vectors spread the disease.

DISCUSSION

Even though the PD of grapes in Taiwan was not reported in a respected journal until this year, it still represents the very first PD in Taiwan as well as in the entire Asian Continent [36]. We did take immediate action from 2002 onward to survey the vineyards using direct isolation and PCR detection and to eradicate diseased grapevines, PD however has not been successfully controlled. In Taiwan, the diseased vineyards were usually in hilly terrains, and therefore it is possible that the vegetation in the vineyards serves as the natural bacterial reservoirs. More than 94 species of plants in 28 families were reported as hosts of PD bacterium in California, and most of them were symptomless hosts. This study represents the first report confirming that *D. palmatus*, *A. brevipedunculata*, *H. scandens* and *M. paniculatus* serve as alternative hosts of *X. fastidiosa* in Taiwan. Because of the availability of the alternative hosts that reserve *X. fastidiosa* in its vegetation, the insect vectors could acquire *X. fastidiosa* at any time and inoculate grapevines. Understanding the stages of *X. fastidiosa* transmission by insect vectors will provide information for PD control in Taiwan.

At last, we should understand thoroughly the epidemiology of PD in order to develop successful control measures. The control strategies include the follows:

(1) Enter Quarantine: The pathogen of PD may be brought in with diseased branches or insect vectors. Therefore, the government must enhance the quarantine of grapevines imported for use as planting material originated from counties where PD occurs and must strongly restrict smuggling.

(2) We should always survey the grapevines for suspicious PD-like symptoms which should be marked up immediately and experts should be contacted for further identification in the high risk area.

(3) Integrated control:

(a) Plant healthy seedlings to prevent the pathogens being brought into the vineyard.

(b) Eradicate the abnormal grapevines immediately.

(c) Monitor the indigenous insect vector population.

(d) Eradicate the confirmed alternative hosts.

(e) Eradicate the weeds that the insect vectors favor.

(f) Pesticides should be applied to the weeds and insects in the vineyards on weekly basis or as recommended. Pesticides with different inhibition mechanisms should be used and changes of pesticides should be done after one type is being used consecutively for 2-3 times.

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Table 1. Survey results of Pierce's disease incidence: total numbers of diseased plants and vineyards in various counties conducted from 2002 to 2012

Country		Diseased grapevine / Diseased vineyard																						
		91 S	91 W	92 S	92 W	93 S	93 W	94 S	94 W	95 S	95 W	96 S	96 W	97 S	97 W	98 S	98 W	99 S	99 W	100 S	100 W	101 S	101 W	Total
Nantou County	Caoton	1/1	9/3	7/5	17/6	16/4	2/2	7/3	8/6	11/3	5/3	6/5	0	19/3	3/2	25/9	15/3	13/7	3/3	13/7	6/2	6/2	2/1	194/29
	Zhushan	0	1/1	5/1	1/1	4/1	0	3/1	0	0	0	30/4	5/2	52/4	1/1	693/29	60/9	221/20	40/4	161/17	18/4	184/17	2/1	1,481/59
	Jiji	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65/1	15/1	0	0	0	0	0	0	80/1
Taichung City	Fengyuan					1/1	0	1/1	0	0	0	0	0	1/1	0	0	0	0	0	1/1	0	0	0	4/3
	Dongshi			7/1	2/1	1/1	0	5/2	0	0	1/1	0	0	1/1	0	0	0	4/1	0	5/1	0	1/1	0	27/4
	Xinshe		2/1	5/2	9/3	0	0	2/2	11/1	25/4	1/1	7/3	0	53/7	11/3	119/13	11/4	106/14	6/2	55/13	1/1	0	2/2	426/26
	Waipu					128/13		84/8	3/1	102/7	28/3	41/7		301/12		160/12		145/16		129/19		7/3		1,128/47
	Houli					187/21		1,341/42		576/38	71/7	501/43		1,649/68		1,732/96		863/57		560/42		166/32		7,313/184
Miaoli County	Zhuolan				4/3	5/2		167/2	121/3	93/2	67/2	20/1	10/2	231/6	22/4	100/6	0	32/3	4/2	39/5	6/2	4/1	2/1	927/15
	Tongxiao					246/9		599/18		107/12		113/9		131/12		150/14		81/9	53/11	268/9		13/3		1,761/31
Total		1/1	12/5	24/9	33/14	588/52	2/2	2,209/79	143/11	914/66	173/17	718/72	15/4	2,438/114	37/10	3,044/180	101/17	1,465/127	106/22	1,231/114	31/9	381/59	8/5	13,674/399

Table 2. Survey results of other possible host plants for *Xylella fastidiosa* Pierce's disease (PD) strains: total number of various plant species collected from the proximity of PD-confirmed vineyards and sample sizes conducted from 2003 to 2012

Country	Vineyard numbers	Plant species / Sample number										
		2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Total
Nantou County												
Caoton	4	44/76	72/210	39/52	11/12	—	11/82	13/78	13/62	12/71	15/81	77/643
Zhushan	3	21/27	56/85	—	—	7/23	10/56	11/89	12/81	9/78	10/67	65/439
Taichung City												
Dongshi	2	69/131	106/290	41/132	—	29/112	—	—	—	—	—	106/665
Houli	13	—	30/73	31/109	70/245	45/142	36/146	38/155	37/172	28/128	26/135	93/1,170
Waipu	5	—	44/85	20/22	44/75	21/59	20/112	22/124	21/131	16/101	17/100	60/709
Xinshe	5	62/112	94/284	—	54/138	35/91	11/78	12/75	13/77	11/69	16/75	72/924
Miaoli County												
Zhuolan	3	—	76/151	39/52	41/149	23/33	10/54	13/55	13/23	10/85	—	68/602
Tongxiao	2	—	35/56	36/83	42/79	17/34	—	—	—	—	—	42/252
Total	37	131/346	205/1234	92/450	127/698	98/494	80/528	80/576	80/546	71/532	67/458	251/5,404

Table 3. Detection of *Xylella fastidiosa* Pierce's disease (PD) strains in four confirmed alternative hosts collected from the proximity of PD-confirmed vineyards conducted from 2003 to 2012

Country	Vineyard numbers	Host plant (positive*/attempted)			
		H. scandens	*A. brevipedunculata*	*D. palmatus*	*M. paniculatus*
Nantou County					
Caoton	4	0/5	0/5	0/11	0/3
Zhushan	3	0/5	3/52	ND	ND
Taichung City					
Dongshi	2	0/8	ND	1/2	ND
Xinshe	5	0/1	0/32	1/27	1/26
Houli	13	1/21	1/19	0/25	0/13
Waipu	5	7/26	4/34	2/14	ND
Miaoli County					
Zhuolan	3	2/4	1/26	1/13	0/8
Tongxiao	2	0/1	ND	ND	0/2
Total		10/71	8/168	5/92	1/52

*: Positive isolation and PCR detection.

ND: Non-detection

Table 4. Source of *Xylella fastidiosa*: Pierce's disease strains from grapevines and other alternative hosts in Taiwan

Species/host or vector	Strains	GenBank accession no.	Source of reference
Xylella fastidiosa			
Grape			
Nantou, Caotun	GV26	JN820134	Su *et al.*, 2013
	GV61	JN990271	Su *et al.*, 2013
Nantou, Zhushan	GV14	JN990274	Su *et al.*, 2013
	GV102	JN990276	Su *et al.*, 2013
Taichung, Houli	GV78	JN831760	Su *et al.*, 2013
	GV84	JN831759	Su *et al.*, 2013
Taichung, Xinshe	GV18	JN990273	Su *et al.*, 2013
	GV20	JN990272	Su *et al.*, 2013
	GV93	JN831758	Su *et al.*, 2013
Miaoli, Zhuolan	GV63	JN935275	Su *et al.*, 2013
	GV103	JN990277	Su *et al.*, 2013
	ATCC35876	DQ991182	Su *et al.*, 2011
	ATCC35879	DQ987477	Su *et al.*, 2011
D. palmatus	DP10	JN990280	This Study
A. brevipedunculata	AB3	JN990278	This Study
	AB4	JN990279	This Study
H. scandens	HS2	JN831755	This Study
	HS3	JN831756	This Study
	HS4	JN831757	This Study
M. paniculatus	MP1	JN831752	This Study
Pear	PLS2	DQ987473	Su *et al.*, 2011
	PE.PLS	AF203392	Mehta and Rosato, 2001
Citrus	CI.52	AF203389	Mehta and Rosato, 2001
Coffee	CO.01	AF203390	Mehta and Rosato, 2001
Mulberry	GHS505	DQ991183	Su *et al.*, 2011
Oleander	GH-9	DQ991185	Su *et al.*, 2011
	Ol	DQ991186	Su *et al.*, 2011
Peach	4-5	DQ991187	Su *et al.*, 2011
Plum	2-4	DQ991188	Su *et al.*, 2011
Pecan	4BD2	DQ991190	Su *et al.*, 2011
Sycamore	SLS55	DQ991193	Su *et al.*, 2011
Xanthomonas axonopodis* pv. *citri			
Citrus	XCW	DQ991194	Su *et al.*, 2011

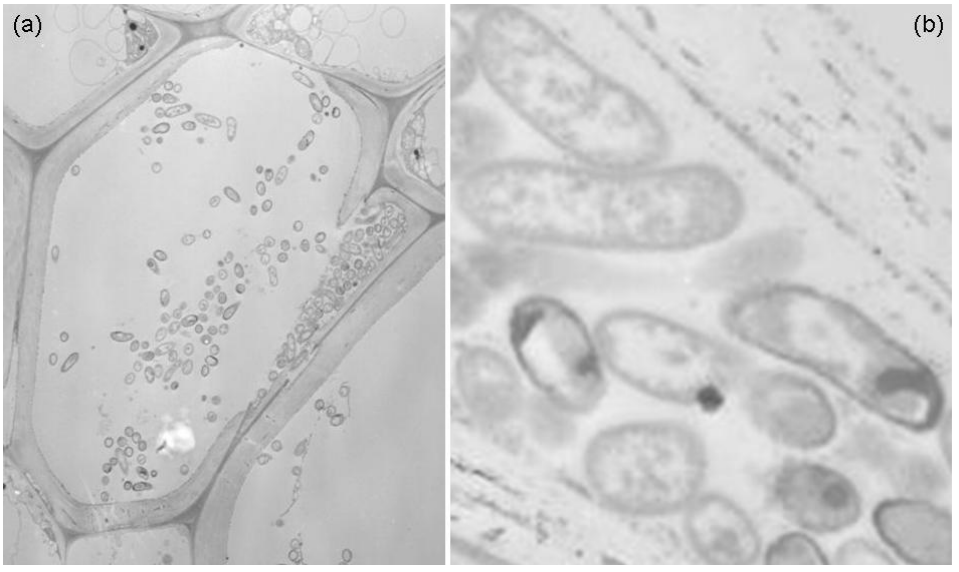

Fig. 1. Cell morphology of Pierce's disease (PD) strains in Taiwan. (a) Transmission electron micrograph of *X. fastidiosa* in the xylem vessel of *D. palmatus* in the vineyard. (b) Enlarged transmission electron micrograph showing cells of *X. fastidiosa* with rippled cell walls.

Fig. 2. (a) Symptoms of PD begins with leaf margin necrosis. (b) Systemic development of leaf scorch (c) Petioles remain attached to the cane (d) Symptoms on the one-year-old grapevine cuttings.

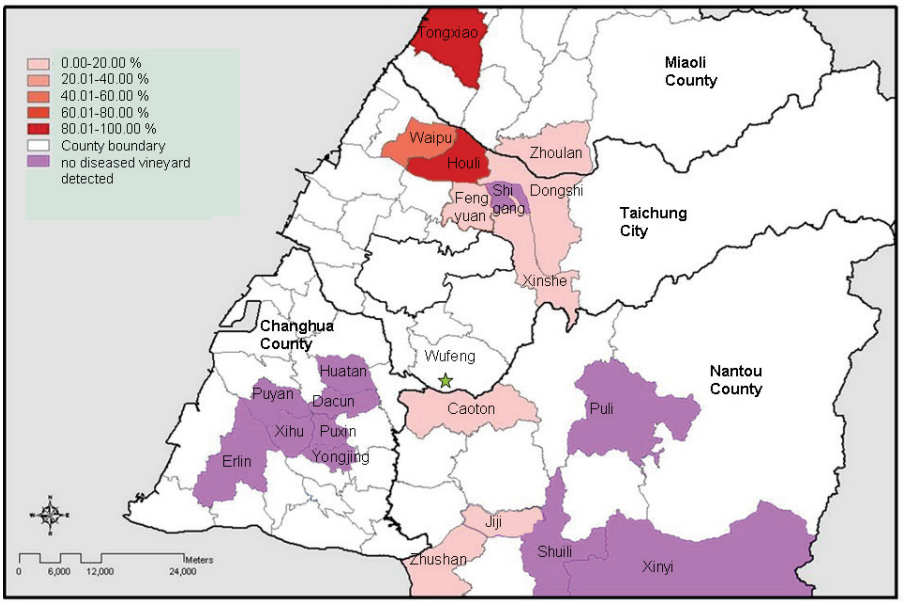

Fig. 3. Survey results of Pierce's disease incident: morbidity of orchards in various counties in central Taiwan conducted from 2002 to 2011.

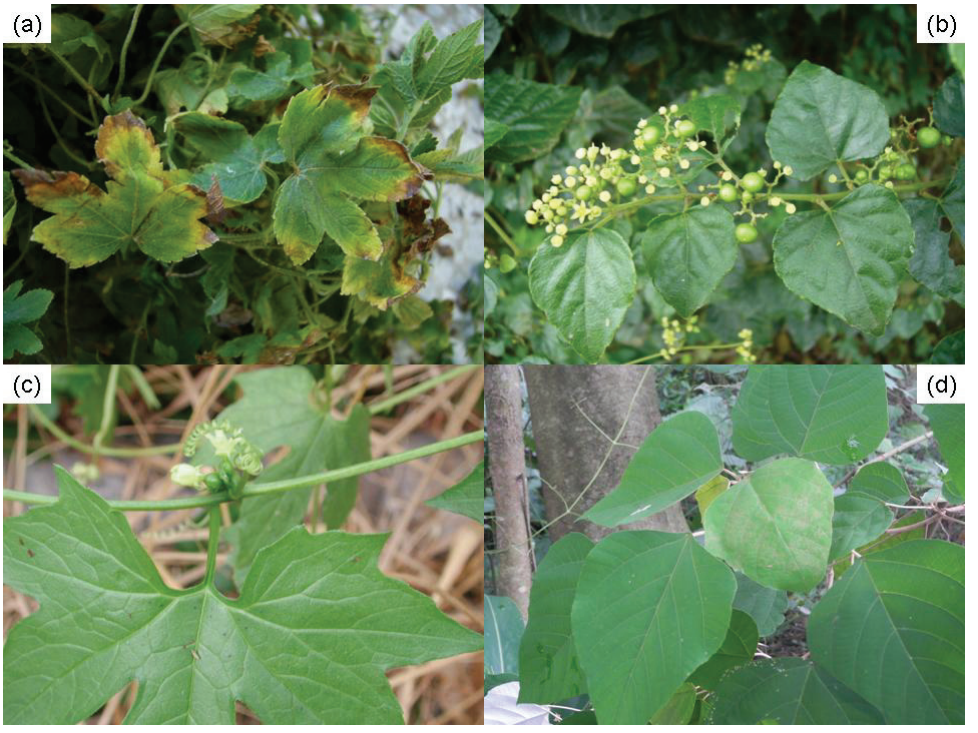

Fig. 4. Four confirmed alternative hosts in Taiwan. (a) *Humulus scandens* (Lour.) Merr.、(b) *Ampelopsis brevipedunculata* (Maxim.) Trautv var. *hancei* (Planch.) Rehder.、(c) *Diplocyclos palmatus* (L.) C. Jeffrey and (d) *Mallotus paniculatus*（Lamm.）Mull. –Arg.

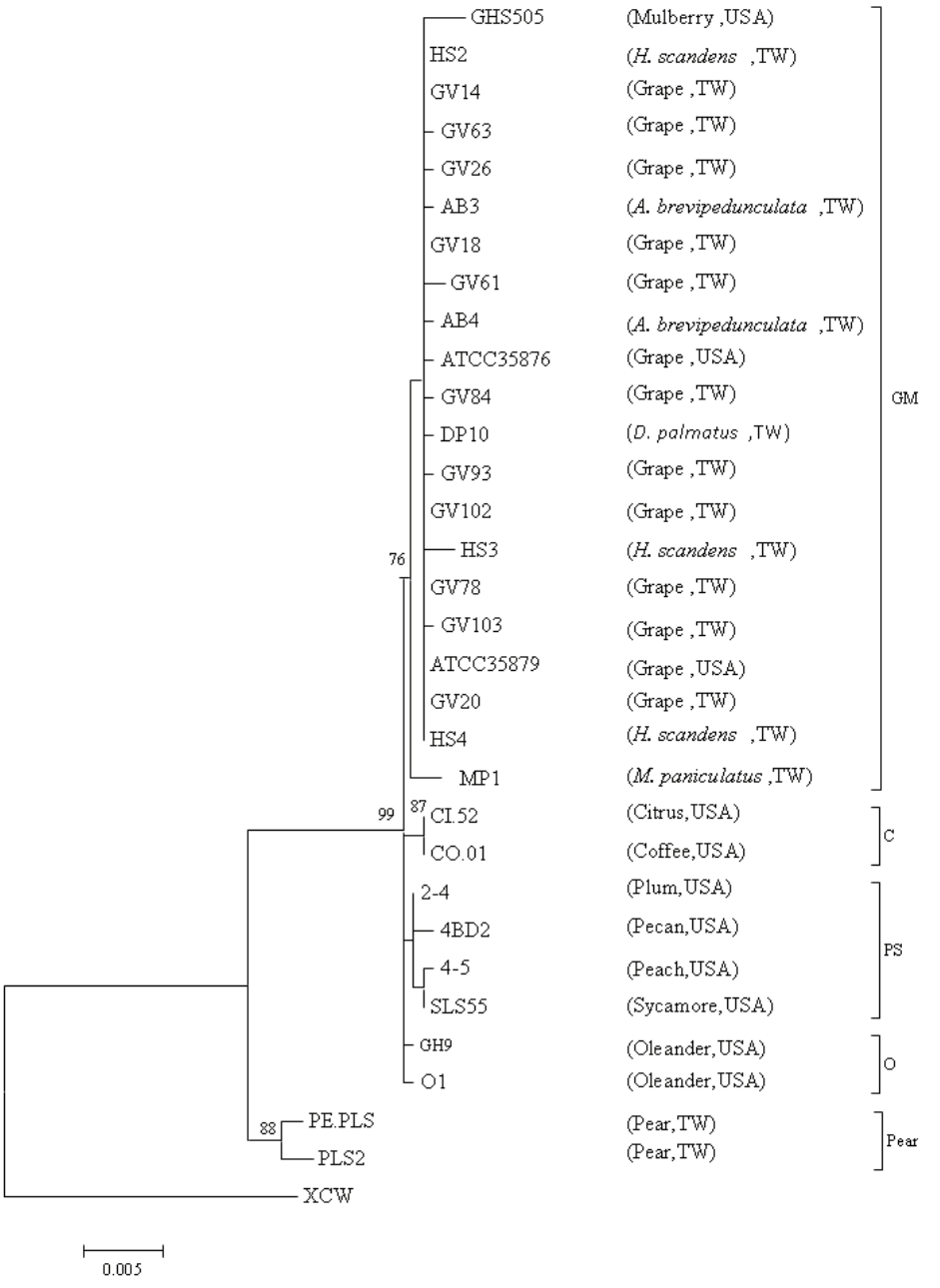

Fig. 5. A neighbour-joining (NJ) tree expressing the relationship of Pierce's disease strains from Taiwan with the other *Xylella fastidiosa* strains based on the 16S rRNA sequence. The tree was rooted using the 16S rRNA gene of *Xanthomonas axonopodis* pv. *citri* as an outgroup. The scale bar is equivalent to 0.05 nucleotide substitution per site. The probabilities of bootstrap analyses (in percentage) for 1000 resamplings that are greater than 70% are shown above the internal branches. The characteristics of the bacterial strains used in this experiment are listed in Table 4.